

Novel Major Bacterial Candidate Division within a Municipal Anaerobic Sludge Digester

Rakia Chouari,¹ Denis Le Paslier,¹ Catherine Dauga,² Patrick Daegelen,¹
Jean Weissenbach,¹ and Abdelghani Sghir^{1*}

CNRS-UMR 8030, Genoscope and Université d'Evry Val d'Essonne, Evry,¹ and
Génopole de l'Institut Pasteur, Paris,² France

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In a previous study, we analyzed the molecular diversity of *Planctomycetales* by PCR amplification and sequencing of 16S rRNA clone libraries generated from a municipal wastewater plant, using planctomycete-specific and universal primer sets (R. Chouari, D. Le Paslier, P. Daegelen, J. Weissenbach, and A. Sghir, *Appl. Environ. Microbiol.* 69:7354–7363, 2003). Only a small fraction (4%) of the 16S rRNA gene sequences of the digester clone library corresponded to the *Planctomycetales* division. Importantly, 85.9% of the digester clone sequences are grouped into two different clusters named WWE1 (81.4% of the sequences) and WWE2 (4.5%) and are distantly affiliated with unidentified bacterial sequences retrieved from a methanogenic reactor community and from a termite gut, respectively. In phylogenetic analysis using 16S rRNA gene sequence representatives of the main phylogenetic bacterial divisions, the two clusters are monophyletic, branch apart from each other, and are distantly related to *Planctomycetales* and other bacterial divisions. A novel candidate division is proposed for WWE1, while the WWE2 cluster strongly affiliates with the recently proposed *Lentisphaerae* phylum. We designed and validated a 16S rRNA probe targeting WWE1 16S rRNA sequences by both fluorescent in situ hybridization (FISH) and dot blot hybridization (DBH). Results of FISH analysis show that WWE1 representative microorganisms are rods or filamentous shaped, while DBH shows that WWE1 accounts for 12% of the total bacterial rRNA within the anaerobic digester. The remaining 16S rRNA gene sequences are affiliated with *Verrucomicrobia* or recently described candidate divisions with no known pure culture representatives, such as OD1, BRC1, or NBL-UPA2, making up less than 3.5% of the clone library, respectively. This inventory expands the known diversity of the latter bacterial division-level lineages.

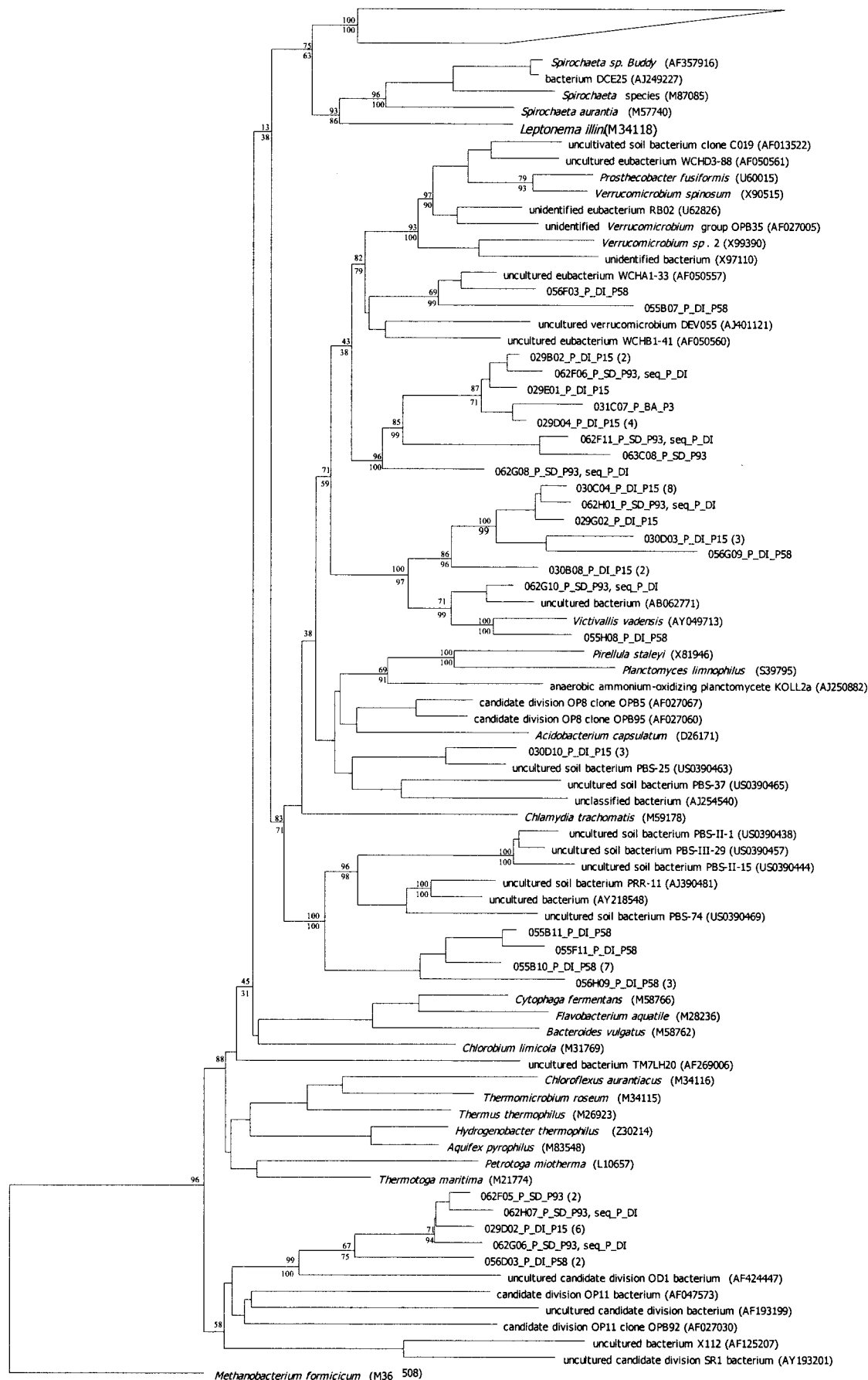
The anaerobic sludge digester ecosystem has been partially described by classical methods (1), and several genera involved in the different steps of the degradation of organic matter to methane and carbon dioxide have been identified. However, to date there has been no comprehensive and exhaustive analysis of the microbial species composition in any anaerobic sludge digester. Despite the use of various media, generally microbiologists have found a remarkable difference in the number and morphology of organisms when comparing natural samples with those obtained from enrichment cultures and isolates from habitat-simulating or selective media. Application of modern molecular approaches, such as rRNA gene sequence analysis, has allowed the unambiguous assignment of cultivated and noncultivated organisms to their nearest phylogenetic neighbor and a better description of the complexity of the ecosystem. The number of bacterial groups has increased from 11 (25) to 50 to 55 main phylogenetic divisions; more than one-third are known only from detection of rRNA sequences and have no described cultivated representatives (18). These molecular inventories are based on the use of universal PCR primers described by Weisburg et al. (24) or Marshesi et al. (15). In many cases, DNA samples extracted from various sources were found to be poor templates for amplification of the 16S rRNA genes with certain so-called universal primers (15). However studies using bacterial division level-specific

primers such as *Planctomycete-Verrucomicrobia*-specific primers revealed a huge hidden diversity at the subdivision level (3, 5). This diversity cannot be described based on the so-called bacterial universal primers, simply because most often these primers have many mismatches with their target in the 16S rRNA gene sequences of some important bacterial divisions.

In the present paper, using both qualitative and quantitative molecular approaches, we describe the occurrence of a novel high-order bacterial phylogenetic lineage never described before within an anaerobic sludge digester. Moreover we confirm the occurrence and extend the diversity of candidate divisions such as OD1, WS3, OP3, NBL-UPA2, and BRC1 previously described on the basis of a few phylotypes only (5, 11).

Anaerobic sludge samples were obtained from an anaerobic mesophilic digester at the Evry wastewater treatment plant (WWTP; 250,000 population equivalents), located about 35 km south of Paris, France. The digester temperature was 33°C, the pH was 7.2, and the digester had 37.5 days of retention time. Samples were centrifuged for 15 min at 20,000 × g. Sludge pellets were washed three times with phosphate-buffered saline and stored at –20°C. Genomic DNA extraction was performed as described by Chouari et al. (3). 16S rRNA genes were amplified from genomic DNA with a *Planctomycetales*-specific forward primer, Pla46F, and a universal reverse primer, 1390R (16). Molecular cloning and sequencing were done as described by Chouari et al. (3). The 16S rRNA gene sequences were treated as described by Ewing et al. (8) and compared with the complete EMBL nucleotide sequence databases. Sequences from EMBL with the best BLAST score

* Corresponding author. Mailing address: CNRS-UMR 8030, Genoscope, 2, rue Gaston Crémieux, 91057 Evry, France. Phone: (33) 1 60 87 25 31. Fax: (33) 1 60 87 25 14. E-mail: sghir@genoscope.cns.fr.



WME1

Spirochaeta

Verrucomicrobia

Lentisphaerae
WWE2

Planctomycetales

OP8

Acidobacteria
NBL-UPA2

OP3

Chlamydia

BRC1

CFB

GSB

TM7

GNSB

Deinococcus

Aquificae

Thermotogae

OD1

OP11

SR1

0.10

were imported into the ARB data set when necessary (<http://www.arb-home.de>). Chimeric sequences were searched by the procedure described by Juretschko et al. (14) prior to phylogenetic analysis. All sequences with more than 1,200 nucleotides were imported into the ARB database and automatically aligned with the existing 16S rRNA gene sequences. The resulting alignments were manually checked and corrected when necessary, and 1,091 unambiguously aligned nucleotide positions were used for phylogenetic analyses. Phylogenetic placement was done in comparison with reference sequences representing the main lines of descent in the domain *Bacteria*, using the ARB program and database package. Tree topology was evaluated by applying neighbor-joining (NJ) analyses with Jukes and Cantor corrections and a 50% invariance criterion for inclusion of individual nucleotide sequence positions in the treeing analyses. We generated several trees which differed in the reference sequences, the set of alignment positions, and the outgroup sequence used. Maximum-parsimony (MP) and maximum-likelihood (ML) methods were also used. The statistical significance levels of interior nodes were determined by performing bootstrap analyses based on 100 resamplings by the NJ and MP methods. Overall, 16S rRNA gene sequence similarities were determined by using the distance matrix tool of the ARB program package (Fig. 1).

To resolve the WWE1 position, a comprehensive set of spirochete 16S rRNA gene sequences was used in a separate analysis (Fig. 2). Aligned sequences were exported from the ARB database and analyzed with PAUP 4.0b10 (Sinauer Associates, Inc., Sunderland, Mass.). For MP analyses, we used a heuristic search strategy, the tree bisection reconnection (TBR) branch-swapping option. A strict consensus tree was drawn when multiple best trees were obtained. For the ML and NJ methods, the evolutionary model was evaluated with MODELTEST 3.0 (17). This test compared our sequence data matrix with various evolutionary models to identify models that best fit the data. We then performed phylogenetic reconstruction based on ML analyses, using the heuristic search strategy with TBR branch swapping. Distance analysis was performed with the same evolutionary model and the NJ algorithm (20). Statistical confidence levels for MP and NJ were evaluated by the nonparametric bootstrap method based on 100 resamplings. Bootstrap analyses for ML analysis were performed without TBR branch swapping to reduce computational time. Nodes represented in more than 90% of bootstrap replicates were considered strongly supported.

The probe search function of the ARB program software package was used to design new 16S rRNA probes, which were tested in silico with the "check probe" function program of RDP (<http://rdp.cme.msu.edu/html/>). For experimental validation of the WWE1-specific probe, since there is no cultivated

microorganism representative of this novel division, 16S rRNA was generated by in vitro transcription of rRNA gene inserts of the WWE1 clones (Riboprobe in vitro transcription system; Promega). Washing temperature determination (T_w) of the new probe was achieved with washing buffer containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) and 1% sodium dodecyl sulfate (SDS).

For dot blot hybridization (DBH) and quantification, total RNA was extracted from approximately 200 mg of six different digester samples (from April to December 2001) by bead beating, phenol extraction, and ethanol-sodium acetate precipitation as described previously (22, 23). The quality of the extracted RNA was checked by formaldehyde gel electrophoresis. Total rRNA was quantitatively normalized with a universal probe as described by Sghir et al. (22). Total rRNA of *Escherichia coli* (Boehringer, Mannheim, Germany) and a transcribed WWE1 rRNA gene were used as an rRNA standard. A total of 200 ng of RNA was blotted onto nylon membranes (Nytran Super Charge; Schleicher and Schuell) in triplicate and hybridized with 32 P-labeled oligonucleotide probes (purchased from Eurogentec, Köln, Germany). A mixture of probes (EUB338, II, and III) was used to quantify specific bacterial rRNA as a percentage of the signal intensity obtained with a universal probe (Beta Imager; Packard Instruments). For the EUB probe mixture, we used 1% SDS and 1% SSC washing buffer under a T_w of 54°C. We determined the experimental T_w for the WWE1-specific probe, and we validated the probe using the same washing buffer composition. WWE1-specific rRNA was quantified and expressed as a percentage of total rRNA measured by EUB I, II, and III as described previously (22).

Fluorescent in situ hybridization (FISH) analyses of sludge samples were done as described by Chouari et al. (3). A stringent wash step was performed for 10 min at 48°C. The newly developed WWE1-specific probe was 5'-end labeled with Cy3 (MWG Biotech, Ebersberg, Germany) and used simultaneously with the fluorescein isothiocyanate-labeled EUB338 (a mixture of probes EUB I, II, and III). We have tested the specificity of the novel probe targeting the novel WWE1 lineage, using pure cultures of *Bacillus firmus* and *Acinetobacter calcoaceticus* as negative controls; no signal was obtained with these microorganisms, whereas they showed a positive signal when the mixture of EUB338 probes was used. The systematic use of unlabeled controls in our experiments confirmed that the bright cocci (Fig. 3A) corresponded with autofluorescence. Slides were visualized with a Zeiss axioplan epifluorescence microscope.

Results obtained from previous work showed important planctomycete diversity in the aerobic and anoxic basins of a municipal WWTP, using a planctomycete-specific and universal primer set. However the 16S rRNA gene clone libraries

FIG. 1. Evolutionary distance dendrogram showing the affiliation of the environmental 16S rRNA gene sequences recovered from the anaerobic digester clone library to representative members of the divisions in the bacterial domain. The dendrogram was reconstructed by the NJ method in combination with a 50% consensus filter for the domain *Bacteria* with the ARB software package. The numbers at the nodes indicate the percentages of recovery of relevant branch points in 100 bootstrap resamplings (values above nodes by NJ, values below nodes by parsimony). The root was determined by using the archaeal 16S rRNA sequence of *Methanobacterium formicicum* (M36508) as the outgroup reference. EMBL, GenBank, and DDBJ accession numbers are given in parentheses. SR1, OP11, OD1, BRC1, OP3, and OP8 represent proposed candidate divisions. The scale bar represents the 10% estimated difference in nucleotide sequence positions.

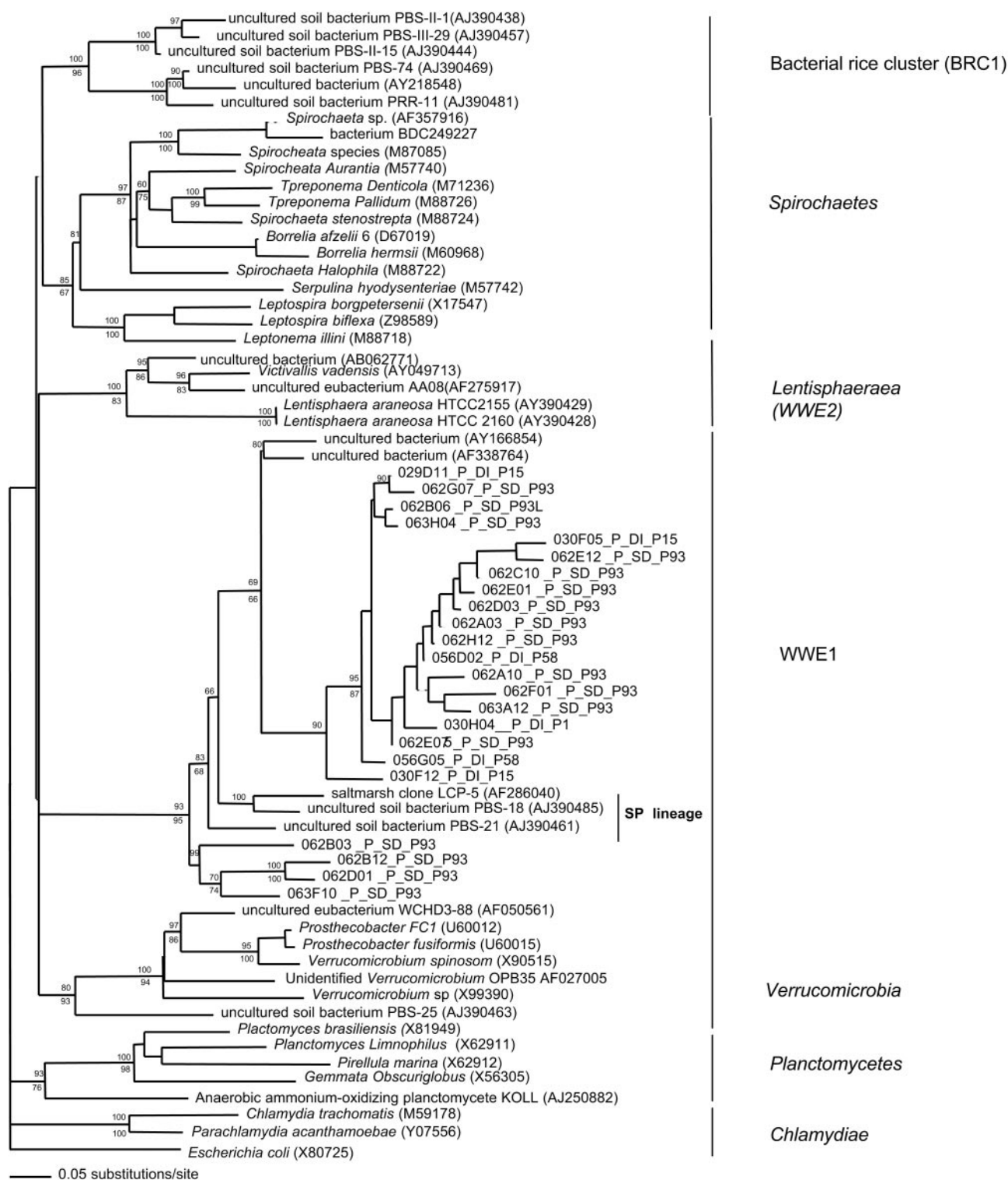


FIG. 2. Evolutionary distance dendrogram constructed by the NJ method showing the affiliation of the environmental WWE1 16S rRNA gene sequences recovered from the anaerobic digester clone library to representative members of the closest divisions in the bacterial domain. A large data set of spirochete 16S rRNA gene sequences was used. Aligned sequences were analyzed by three methods (NJ, MP, and ML) provided by PAUP 4.0b10 as described in the text. The numbers at the nodes indicate the percentages of recovery of relevant branch points in 100 bootstrap resamplings (values above nodes by NJ, values below nodes by parsimony). The *E. coli* 16S rRNA gene sequence was used as the outgroup to define the root of the tree. The scale bar represent the 5% estimated difference in nucleotide sequence positions.

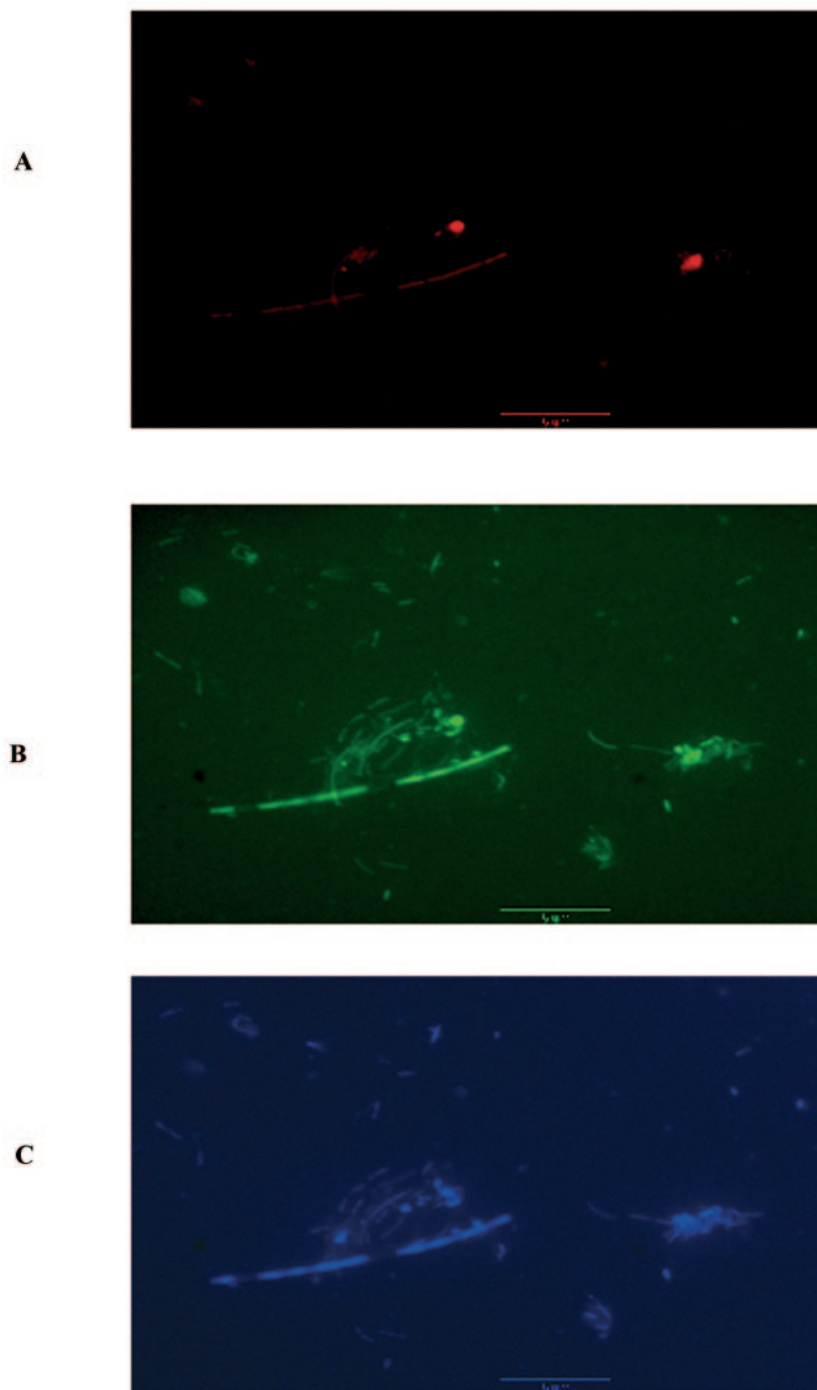


FIG. 3. In situ identification of the WWE1 candidate division in an anaerobic municipal sludge digester, using Cy3-labeled WWE1-specific probe S^{*}-WWE1-1181-a-A-18. (A) Cells identified, marked with WWE1-specific Cy3-labeled probe. (B) EUB338, II, and III are green. (C) DAPI (4',6'-diamidino-2-phenylindole) staining. The bright cocci in panel A represent autofluorescence. Microscopic visualization was achieved with a Zeiss axioplan epifluorescence microscope.

generated from the anaerobic digester using the same primer set yielded a total of 396 rRNA gene sequences, of which only 4% of the clone libraries are affiliated with planctomycetes (3) (Table 1). The remaining 96% are affiliated with uncultured bacterial sequences and show a higher degree of diversity. We

recovered 16S rRNA gene sequence types, which could not be assigned to the *Planctomycetales* but to two novel bacterial predominant groups we named WWE1 and WWE2 (for wastewater of Evry), represented by 85.9% (340 of 396 clones) and 4.5% (18 of 396 clones) of the clone library, respectively. Other

TABLE 1. Distribution of clone sequences and OTUs analyzed in the present study^a

Phylum and candidate division	No. (%) of clones	No. (%) of OTUs	% of intradivergence
<i>Planctomycetales</i>	16 (4.0)	8 (9.2)	
WWE1	322 (81.4)	52 (59.8)	29
WWE2	18 (4.5)	8 (9.2)	19
<i>Verrucomicrobiales</i>	13 (3.3)	9 (10.3)	22
OD1	12 (3.0)	5 (5.7)	33
BRC1	12 (3.0)	4 (4.6)	24
NBL-UPA2	3 (0.8)	1 (1.2)	18.4
Total	396 (100)	87 (100)	

^a The number of OTUs was calculated with a threshold value of 97% 16S rRNA gene similarity (9).

16S rRNA gene sequences are affiliated with the *Verrucomicrobia*, considered by Woese (25) as bacterial sister groups, and some other novel candidate divisions recently described by several authors, such as OD1 (11) or BRC1 and NBL-UPA2 (5).

WWE1 and WWE2 are distantly related to 16S rRNA gene sequences of cultivated bacteria (Table 2 and Fig. 1). WWE2 exhibits a high-order diversity of rRNA gene sequences and is distantly affiliated with an unidentified 16S rRNA gene sequence (AB062771) retrieved from a termite gut (*Coptotermus formosus*) with sequence homology ranging between 81.6 and 96.6%. This group of 18 16S rRNA gene sequences is monophyletic, encompassing a recently cultured bacterium, *Victivallis vadensis*, isolated from human feces (26). The authors placed this bacterium among a deeply branching group within the *Verrucomicrobiales*, but the present phylogenetic analysis using more closely related 16S rRNA gene sequences shows the WWE2 group branching independently from the *Verrucomicrobia* in all types of treeing analysis. The confidence node of WWE2 16S rRNA gene sequences is supported by a strong bootstrap value ($\geq 97\%$). WWE2 seems to form a novel high-order phylogenetic cluster within the *Bacteria* domain comparable to *Planctomycetes* or *Verrucomicrobia*. They can be considered as a *Planctomycetes*, *Verrucomicrobia*, or *Chlamydia* sister group. Our results are corroborated by recent results of Cho et al. (2) showing that *Victivallis vadensis* could be rather placed within the new phylogenetic phylum of *Lentisphaerae* (Fig. 1). Thus, WWE2 16S rRNA gene sequences are strongly associated with this new phylum.

WWE1 encompasses sequences affiliated with an unidentified clone 16S rRNA gene sequence of a clone retrieved from a methanogenic reactor (AY166854) (80.2 to 92.6% sequence similarity) (7) (Fig. 1 and Table 2). Phylogenetic analysis performed by Gu et al. (10) placed the latter clone sequence among a number of unidentified sequences deeply branching among a group of cultivated *Spirochaetes* 16S rRNA gene sequences, with $\leq 80\%$ sequence homology. The authors' phylogenetic analysis was based on a small selection of 16S rRNA gene sequences. WWE1 also encompasses two 16S rRNA gene sequences (AJ390461 and AJ390485) retrieved from anoxic bulk soil of a flooded rice microcosm (5). In their phylogenetic analyses, Derakshani and coworkers showed that the two sequences cluster together in a monophyletic cluster they called SP lineage (with reference to spirochetes) showing distinct

affiliation with the spirochetes with an overall level of 16S rRNA gene sequence dissimilarity ranging between 23 to 27%. If *Leptospira* spp., which form a deeply branching sublineage of spirochetes, are included in the phylogenetic analyses, the bootstrap support for assignment of SP lineage to the spirochete drops from 93% to 40 to 60%. In our study, we enriched this group of 16S rRNA gene sequences by 322 sequences. We used a comprehensive number of cultivated spirochete 16S rRNA gene sequences and included sequences from reports by Derakshani et al. (5) and Gu et al. (10); using the three treeing methods, phylogenetic analyses indicate that WWE1 and *Spirochaetes* are clearly making two monophyletic and distinct clusters (Fig. 2). We show that sequences from the reports by Derakshani or Gu et al., along with our WWE1 rRNA gene sequences, cluster in a monophyletic lineage with a bootstrap value of $>93\%$. All treeing analyses using a number of different bacterial division sequence representatives show that WWE1 is a monophyletic lineage branching distinctly from *Spirochaetes* and other main lines of bacterial descent. Thus, WWE1 appears as a *Spirochaetes* division sister group, clearly distinguishable and forming a completely independent lineage (Fig. 2). 16S rRNA gene sequence dissimilarity between WWE1 and adjacent phyla (*Lentisphaerae*, *Verrucomicrobia*, and *Planctomycetes*; BRC1) is more than 20%. Sequence dissimilarity with the *Spirochaetes* phylum ranged between 20.9 and 28.7%. Hugenholtz et al. (12) suggested that 85% sequence identity be used as a cutoff for distinguishing new phyla; thus, by this criterion together with phylogenetic position, this clade was considered to be a novel phylum-level lineage in the domain *Bacteria*.

Comparison of 16S rRNA sequence signatures which distinguish among *Bacteria*, *Archaea*, and *Eukarya* was performed as described by Woese (25). Sequence signatures of WWE1 were clearly related to *Bacteria* and might be considered as *Spirochaetes* and *Verrucomicrobia* sister groups, respectively (data not shown). The percentage of intradivergence within WWE1 lineage is 29%, which is comparable to that of other well-established divisions (6).

Representative sequences of these lineages have also been retrieved from two other anaerobic digesters, such as the Corbeil (2 km upstream of Evry) and Creil (100 km downstream of Evry) WWTPs. They represented 14.7 and 19.2% of the generated clone libraries (data not shown). This means that microorganisms harboring such 16S rRNA gene sequences are environmentally widely distributed. They might be important components of the trophic chain of degradation of the organic matter under anaerobic conditions.

Quantitative DBH and FISH techniques are providing quantitative information on the abundance of a microorganism or a group of microorganisms, their shape and their spatial localization within the original environmental setting. We developed a WWE1-specific probe, S-*₁-WWE1-1181-a-A-18 (Table 3). The washing temperatures for the WWE1 probe was determined to be 50°C, using a washing buffer containing 1× SSC and 1% SDS as described by Sghir et al. (21, 22). Application of the probe using DBH on six different total RNA extracts from anaerobic digester samples showed that WWE1 rRNA represents $11.9\% \pm 3.1\%$ of the total bacterial rRNA, which is comparable to the relative rRNA index of a bacterial phylum like *Proteobacteria*, represented by 14% of total rRNA (unpub-

TABLE 2. Affiliation of the bacterial 16S rRNA gene sequences analyzed in this study

Group by similarity in OTU database ^a	No. of clones	Closest sequence/microorganism	Accession no.	% Similarity
WWE1				
063H04_P_SD_P93	1	Uncultured bacterium	AY166854	92.6
063H06_P_SD_P93	1	Uncultured bacterium	AY166854	91.7
063H06_P_SD_P93	1	Uncultured bacterium	AY166854	91.7
056E03_P_DI_P58	1	Uncultured bacterium	AY166854	91.3
062B06_P_SD_P93	1	Uncultured bacterium	AY166854	90.7
056B03_P_DI_P58	4	Uncultured bacterium	AY166854	90.7
056F06_P_DI_P58	55	Uncultured bacterium	AY166854	90.5
030D05_P_DI_P15	1	Uncultured bacterium	AY166854	90.3
062A03_P_SD_P93	1	Uncultured bacterium	AY166854	90.2
029D03_P_DI_P15	3	Uncultured bacterium	AF338764	90.2
030H04_P_DI_P15	8	Uncultured bacterium	AY166854	90.1
056C06_P_DI_P58	1	Uncultured bacterium	AY166854	90.1
056G05_P_DI_P58	1	Uncultured bacterium	AY166854	90.1
062H10_P_SD_P93	1	Uncultured bacterium	AY166854	89.9
062A11_P_SD_P93	3	Uncultured bacterium	AY166854	89.9
062F10_P_SD_P93	1	Uncultured bacterium	AY166854	89.9
062H12_P_SD_P93	1	Uncultured bacterium	AY166854	89.8
062G07_P_SD_P93	1	Uncultured bacterium	AF338764	89.7
062E07_P_SD_P93	1	Uncultured bacterium	AY166854	89.6
056D02_P_DI_P58	1	Uncultured bacterium	AY166854	89.6
063B07_P_SD_P93	1	Uncultured bacterium	AY166854	89.6
029D11_P_DI_P15	12	Uncultured bacterium	AY166854	89.5
062G03_P_SD_P93	2	Uncultured bacterium	AY166854	89.5
029H08_P_DI_P15	29	Uncultured bacterium	AY166854	89.4
063D01_P_SD_P93	1	Uncultured bacterium	AY166854	89.2
062E11_P_SD_P93	95	Uncultured bacterium	AY166854	89.2
029C01_P_DI_P15	1	Uncultured bacterium	AY166854	89.1
029C09_P_DI_P15	6	Uncultured bacterium	AY166854	89.0
030C02_P_DI_P15	1	Uncultured bacterium	AY166854	88.9
030F05_P_DI_P15	2	Uncultured bacterium	AY166854	88.9
030F11_P_DI_P15	1	Uncultured bacterium	AY166854	88.9
062C10_P_SD_P93	3	Uncultured bacterium	AY166854	88.7
062D07_P_SD_P93	3	Uncultured bacterium	AY166854	88.6
062F08_P_SD_P93	4	Uncultured bacterium	AY166854	88.2
063D08_P_SD_P93	1	Uncultured bacterium	AY166854	88.2
063A07_P_SD_P93	1	Uncultured bacterium	AY166854	88.2
062A04_P_SD_P93	2	Uncultured bacterium	AY166854	87.8
062D11_P_SD_P93	54	Uncultured bacterium	AY166854	87.8
062A10_P_SD_P93	1	Uncultured bacterium	AY166854	87.6
062E01_P_SD_P93	1	Uncultured bacterium	AY166854	87.5
062D03_P_SD_P93	1	Uncultured bacterium	AY166854	87.1
062E12_P_SD_P93	1	Uncultured bacterium	AY166854	87.1
063F10_P_SD_P93	1	Uncultured bacterium	AB062771	86.3
030F12_P_DI_P15	1	Uncultured bacterium	AY166854	86.3
063F05_P_SD_P93	1	Uncultured bacterium	AY166854	85.7
062F01_P_SD_P93	1	Uncultured bacterium	AY166854	85.5
063A12_P_SD_P93	1	Uncultured bacterium	AY166854	85.4
063H02_P_SD_P93	1	Uncultured bacterium	AY166854	84.5
063F12_P_SD_P93	1	Uncultured bacterium	AB074941	83.4
062D01_P_SD_P93	1	Uncultured bacterium	AB074941	82.1
062B12_P_SD_P93	2	Uncultured bacterium	AB074941	81.2
062B03_P_SD_P93	1	Uncultured bacterium	AB074941	80.2
Total, 52	322			
BRC1				
055B10_P_DI_P58	7	Uncultured soil bacterium	AJ390481	85.6
055B11_P_DI_P58	1	Uncultured soil bacterium	AJ390481	83.7
055F11_P_DI_P58	1	Uncultured soil bacterium	AJ390481	82.7
056H09_P_DI_P58	3	Uncultured soil bacterium	AJ390481	77.9
Total, 4	12			
OD1				
062G06_P_SD_P93	1	Uncultured bacterium	AF424447	79.6
029D02_P_DI_P15	6	Uncultured bacterium	AF424447	79.2
062F05_P_SD_P93	2	Uncultured bacterium	AF424447	78.3
056D03_P_DI_P58	2	Uncultured bacterium	AF424447	77.5

Continued on following page

TABLE 2—Continued

Group by similarity in OTU database ^a	No. of clones	Closest sequence/microorganism	Accession no.	% Similarity
062H07_P_SD_P93	1	Uncultured bacterium	AF424447	77.4
Total, 5	12			
<i>Verrucomicrobia</i>				
056F03_P_DI_P58	1	Uncultured eubacterium	AF050557	94.9
055B07_P_DI_P58	1	Uncultured eubacterium	AF050557	89.1
029D04_P_DI_P15	4	Uncultured eubacterium	AF050560	83.5
062F06_P_SD_P93	1	Unidentified eubacterium	U62926	82.5
029E01_P_DI_P15	1	Unidentified eubacterium	U62926	82.6
029B02_P_DI_P15	2	Uncultured soil bacterium	AJ390455	82.9
062G08_P_SD_P93	1	Unidentified eubacterium	U62837	82.4
062F11_P_SD_P93	1	Uncultured eubacterium	AF050557	80.6
063C08_P_SD_P93	1	Uncultured eubacterium	AF050557	80.7
Total, 9		13		
<i>Lentisphaerae</i> (WWE2)				
062G10_P_SD_P93	1	Uncultured bacterium	AB062771	96.6
055H08_P_DI_P58	1	<i>Victivallis vadensis</i>	AY049713	93.2
030B08_P_DI_P15	2	Uncultured bacterium	AB062771	86.5
030C04_P_DI_P15	8	Uncultured bacterium	AB062771	84.5
029G02_P_DI_P15	1	Uncultured bacterium	AB062771	84.7
062H01_P_SD_P93	1	Uncultured bacterium	AB062771	84.8
030D03_P_DI_P15	3	Uncultured bacterium	AB062771	82.5
056G09_P_DI_P58	1	Uncultured bacterium	AB062771	81.6
Total, 8	18			
NBL-UPA2				
030D10_P_DI_P15	3	Uncultured soil bacterium	AJ390463	90.6
Total, 1	3			
Overall total, 79	380			

^a Clones with a 16S rRNA gene sequence similarity of $\geq 97\%$ to each other were grouped into an OTU.

lished data). The same probe was Cy3-5'-end labeled and used to track representatives of WWE1 within the digester samples by the FISH technique. Results show that the probe binds to rods and filamentous shaped microorganisms but not spiral

shaped organisms, which is a characteristic of the *Spirochaetes* phylum (Fig. 3). Similar results were obtained by Rossetti et al. (19) using the SPL998 16S rRNA probe, which targets a set of sequences affiliated with the WWE1 lineage (data not shown).

TABLE 3. Novel candidate division-specific probe S*-WWE1-1181-a-A-18 based on the 5'→3' 16S rRNA sequence alignment

Clone or environmental sequence (accession no.) ^a	Sequence ^b
Probe S*-WWE1-1181-a-A-18.....	5' CTTCTCTGCGTTGTTAC 3'
Target.....	3' GAAGGAGACGCAACAATG 5'
55F12_P_DI_P58.....	5' ----- 3'
Uncultured bacterium (AF338764).....	5' ----- 3'
Uncultured bacterium (AB062771).....	5' --GUU--GCAG----- 3'
030C04_P_DI_P15 (WWE1; this study).....	5' --GU--GCGG----- 3'
<i>Pirellula staleyi</i> (X81946).....	5' --GUU--A----- 3'
<i>Acidobacterium capsulatum</i> (D26171).....	5' CCG-U----- 3'
<i>Escherichia coli</i>	5' --G-U--ACUG----- 3'
<i>Acinetobacter calcoaceticus</i> (X81657).....	5' --G---ACUG----- 3'
<i>Bacillus firmus</i> (D78314).....	5' --G---AC-G----- 3'

^a The probe designation is in accordance with the Oligonucleotide Probe Database nomenclature.

^b Sequence for positions 1164 to 1181 (*E. coli* numbering). Dashes indicate identity with the target sequence. There were at least four mismatches for all nontarget sequences.

This provides further support for considering this major lineage as a candidate division, which may be recovered from methanogenic environments. The novel probe described in this study will enable us to determine the distribution and population dynamics of the WWE1 lineage in environmental samples such as sludge digesters and termite, animal, or human gut.

Sequences affiliated with other bacterial divisions such as the *Verrucomicrobiales*, OD1, BRC1, and NBL-UPA2 were also found in the clone library (Table 1 and Fig. 1). For the *Verrucomicrobia*, the phylogenetic tree depicted in Fig. 1 shows that besides the five *Verrucomicrobia* subdivisions defined by Hugenholtz et al. (13), a novel subdivision might be defined. Sequence similarity of all of those *Verrucomicrobia* sequences to the most closely affiliated 16S rRNA gene sequence ranges between 80.7 and 94.9%. The intradivergence of 20% known for this division increased with the newly characterized sequences to 22%. The novel sequences also show at least three mismatches with probe EUB338 III, which was designed for *Verrucomicrobiales* (4).

Twelve 16S rRNA gene sequences were grouped within a recently described candidate division, OD1 (11). Sequence similarity of OD1 rRNA gene sequences to known environmental sequences ranges between 77.4 and 79.6%. The BRC1 candidate division proposed on the basis of 11 sequences by Derakshani et al. (5) is represented by 12 sequences defining four operational taxonomic units (OTUs), showing between 77.9 and 85.6% similarity with the most closely affiliated uncultured soil bacterium AF390481 (Fig. 1). Thirteen more BRC1 sequences were recovered from aerobic and anoxic basins (data not shown). Other sequences were found to be affiliated with the candidate division NBL-UPA2 (5), represented by three sequences defining one OTU. Five more sequences were retrieved from the aerobic and anoxic basins (data not shown); they cluster together and show 90.6% similarity to the uncultured soil bacterium AJ390463. They extend this recently described candidate division and thus confirm the existence of this novel bacterial lineage (Fig. 1).

Due to the limitation imposed by the so-called universal primers, we explored a novel fruitful strategy based on the use of a specific division-level primer set for assessment of the diversity of quantitatively important but less known bacterial divisions. Many unique lineages were sampled within the digester ecosystem by using a specific primer set targeting the *Planctomycetales* division. These groups may constitute novel divisions or subphyla, greatly expanding the known higher-order diversity of *Bacteria*. The discovery of division or subphylum phylogenetic lineages is a step toward exploring the relationship between bacterial diversity and biogeochemical function within the digester ecosystems. However, further elucidation of their role in such complex ecosystem may require new culturing approaches or metagenomic studies that allow the linkage of functional and rRNA genes.

Nucleotide sequence accession number. Sequences reported in this study have been submitted to the EMBL, GenBank, and DDBJ databases under accession numbers CR933019 to CR933097.

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REFERENCES

1. Archer, D. B., and B. H. Kirsop. 1990. The microbiology and control of anaerobic digestion, p. 43–89. In A. Wheatley (ed.), *Anaerobic digestion: a waste treatment technology*. Elsevier Science Publishing Ltd., London, England.
2. Cho, J. C., K. L. Vergin, R. M. Morris, and S. J. Giovannoni. 2004. *Lentisphaera araneosa* gen. nov., sp. nov., a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, *Lentisphaerae*. *Environ. Microbiol.* 6:611–621.
3. Chouari, R., D. Le Paslier, P. Daegelen, P. Ginestet, J. Weissenbach, and A. Sghir. 2003. Molecular evidence for novel planctomycete diversity in a municipal wastewater treatment plant. *Appl. Environ. Microbiol.* 69:7354–7363.
4. Daims, H., A. Bruhl, R. Amann, K. H. Schleifer, and M. Wagner. 1999. The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* 22:434–444.
5. Derakshani, M., T. Lukow, and W. Liesack. 2001. Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Appl. Environ. Microbiol.* 67:623–631.
6. Dojka, M. A., J. K. Harris, and N. R. Pace. 2000. Expanding the known diversity and environmental distribution of an uncultured phylogenetic division of bacteria. *Appl. Environ. Microbiol.* 66:1617–1621.
7. Dollhopf, S. L., S. A. Hashsham, and J. M. Tiedje. 2001. Interpreting 16S rDNA T-RFLP data: application of self-organizing maps and principal component analysis to describe community dynamics and convergence. *Microb. Ecol.* 42:495–505.
8. Ewing, B., and P. Green. 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* 8:186–194.
9. Goebel, B. M., and E. Stackebrandt. 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl. Environ. Microbiol.* 60:1614–1621.
10. Gu, A. Z., B. P. Hedlund, J. T. Staley, S. E. Strand, and H. D. Stensel. 2004. Analysis and comparison of the microbial community structures of two enrichment cultures capable of reductively dechlorinating TCE and cis-DCE. *Environ. Microbiol.* 6:45–54.
11. Harris, J. K., S. T. Kelley, and N. R. Pace. 2004. New perspective on uncultured bacterial phylogenetic division OP11. *Appl. Environ. Microbiol.* 70:845–849.
12. Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180:4765–4774. (Erratum, 180:6793.)
13. Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. *J. Bacteriol.* 180:366–376.
14. Juretschko, S., A. Loy, A. Lehner, and M. Wagner. 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. *Syst. Appl. Microbiol.* 25:84–99.
15. Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* 64:795–799.
16. Neef, A., R. Amann, H. Schlesner, and K. H. Schleifer. 1998. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology* 144:3257–3266.
17. Posada, D., and K. A. Crandall. 2001. Selecting models of nucleotide substitution: an application to human immunodeficiency virus 1 (HIV-1). *Mol. Biol. Evol.* 18:897–906.
18. Rappe, M. S., and S. J. Giovannoni. 2003. The uncultured microbial majority. *Annu. Rev. Microbiol.* 57:369–394.
19. Rossetti, S., L. L. Blackall, M. Majone, P. Hugenholtz, J. J. Plumb, and V. Tandoi. 2003. Kinetic and phylogenetic characterization of an anaerobic dechlorinating microbial community. *Microbiology* 149:459–469.
20. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.
21. Sghir, A., D. Antonopoulos, and R. I. Mackie. 1998. Design and evaluation of a *Lactobacillus* group-specific ribosomal RNA-targeted hybridization probe and its application to the study of intestinal microecology in pigs. *Syst. Appl. Microbiol.* 21:291–296.
22. Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Dore. 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 66:2263–2266.
23. Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* 54:1079–1084.
24. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173:697–703.
25. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 51:221–271.
26. Zoetendal, E. G., C. M. Plugge, A. D. Akkermans, and W. M. de Vos. 2003. *Victivallis vadensis* gen. nov., sp. nov., a sugar-fermenting anaerobe from human faeces. *Int. J. Syst. Evol. Microbiol.* 53:211–215.